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CD8$^+$ T cell immunodominance shifts during the early stages of acute LCMV infection independently from functional avidity maturation

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ABSTRACT

Virus-specific T cell responses are often directed to a small subset of possible epitopes and their relative magnitude defines their hierarchy. We determined the size and functional avidity of 4 representative peptide-specific CD8$^+$ T cell populations in C57BL/6 mice at different time points after lymphocytic choriomeningitis virus (LCMV) infection. We found that the frequency of different peptide-specific T cell populations in the spleen changed independently over the first 8 days after infection. These changes were not associated with a larger or more rapid increase in functional avidity and yet still resulted in a shift in the final immunodominance hierarchy. Thus, the immunodominance observed at the peak of an antiviral T cell response is not necessarily determined by the initial size or rate of functional avidity maturation of peptide-specific T cell populations.

Introduction

Although a pathogen may encode a large number of potential peptides that can bind major histocompatibility complex class I (MHC Class I) molecules, often only a limited number of peptide antigens are recognized by CD8$^+$ T cells (Chen and McCluskey, 2006; Yewdell and Bennink, 1999). These CD8$^+$ T cell responses can be ranked by the size of each peptide-specific CD8$^+$ T cell population to define an immunodominance hierarchy. Strong T cell responses are defined as dominant, whereas weak responses are considered subdominant. Multiple factors determine the immunodominance hierarchy and elucidation of these factors is important in the design of effective vaccines, which should induce T cell responses to a broad array of antigens in order to prevent escape of a pathogen by mutation of any single epitope (Phillips et al., 1991; Pircher et al., 1990). Studies have shown that the formation of immunodominance hierarchies can be influenced or associated with changes in antigen processing and transport (Chen et al., 2000; Crowe et al., 2003), the affinity of the peptide for MHC (Chen et al., 2000; van der Most et al., 1997), the avidity of the TcR for peptide:MHC (Zehn et al., 2009), the size (Butz and Bevan, 1998; Choi et al., 2002; Obar et al., 2008) and repertoire (Daly et al., 1995) of the precursor T cell population, and competition between T cell populations (Brehm et al., 2002; Rodriguez et al., 2002) with cytokines such as IFNγ appearing to play an early role in this process (Liu et al., 2004; Rodriguez et al., 2002). Thus, it is likely that most immunodominance hierarchies are determined by the combined impact of several independent factors.

Lymphocytic choriomeningitis virus (LCMV) infection of mice is an established model for the study of antiviral CD8$^+$ T cell responses (Buchmeier and Zajac, 1999; Oldstone 2002). The infection is typically asymptomatic but results in strong antiviral T cell responses that can be monitored directly *ex vivo* using intracellular cytokine staining after peptide stimulation (Homann et al., 2001; Murali-Krishna et al., 1998; Slifka and Whitton, 2001). LCMV-specific T cell responses can be detected as early as 3 days after infection (Homann et al., 2001; Murali-Krishna et al., 1998) and peak by 7–8 days after infection (Homann et al., 2001; Murali-Krishna et al., 1998; Raue and Slifka, 2007) after which the response declines by 10- to 20-fold before reaching a stable memory phase (Grayson et al., 2002; Homann et al., 2001; Murali-Krishna et al., 1998; Raue and Slifka, 2007; Tebo et al., 2005). CD8$^+$ T cells from LCMV-infected BALB/c mice recognize several peptide epitopes (NP118, NP313, GP99, GP283) with 90–95% of LCMV-specific CD8$^+$ T cells focused on the immunodominant peptide, NP118 (van der Most et al., 1997; van der Most et al., 1998). NP118-specific CD8$^+$ T cells increase their sensitivity to peptide antigen by up to 80-fold (Slifka and Whitton, 2001) in a process described as functional avidity maturation. CD8$^+$ T cells from LCMV-infected C57BL/6 mice recognize up to 28 different peptides (Kotturi et al., 2007). CD8$^+$ T cells specific for two peptides, NP396 and GP33/34 (a peptide presented by H-2D^b and H-2K^b), dominate the response with the remainder of the antiviral CD8$^+$ T cells representing either intermediate or subdominant T cell responses. In this study, we selected 4 representative peptides (NP396, GP33/34, GP276, NP205) recognized by CD8$^+$ T cells from LCMV-infected C57BL/6 mice and evaluated the magnitude and functional avidity of the peptide-specific responses in the spleen at early and late time points after infection using direct *ex vivo* peptide stimulation and intracellular cytokine

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staining analysis. During the acute phase of infection, changes in the frequency of the peptide-specific CD8$^+$ T cell populations resulted in a previously unrealized but reproducible shift in the immunodominance hierarchy. This result was unrelated to changes in the functional avidity of the individual CD8$^+$ T cell responses and, notably, not all T cell populations underwent extensive functional avidity maturation. Moreover, the early shift in immunodominance indicates that the final immunodominance hierarchy evolves rapidly during acute infection and is not strictly determined by the size of the precursor T cell populations.

Results

Immunodominance after LCMV infection

We assessed the immunodominance hierarchy of GP33/34-, NP396-, GP276- and NP205-specific CD8$^+$ T cells at different time points after infection with LCMV-Armstrong using direct *ex vivo* peptide stimulation followed by intracellular cytokine staining for IFNγ (Fig. 1). Initially (day 4.5), the combined GP33/34-specific T cell response was approximately 2-fold greater than the NP396-specific T cell response. This might be expected since the naïve T cell precursor frequency for GP33/H-2D^b is estimated to be about twice the size of the naïve NP396/H-2D^b-specific CD8$^+$ T cell population (Obar et al., 2008). The frequency of each peptide-specific CD8$^+$ T cell response in the infected spleen approximately doubled between 4.5 and 5 days after infection, consistent with the rapid proliferation rates observed during the early stages of LCMV infection (Homann et al., 2001; Murali-Krishna et al., 1998). In contrast, between 5 and 8 days after infection the expansion of these responses was no longer synchronized, resulting in a gradual shift in the immunodominance hierarchy. The NP396-specific T cell response was subdominant to the combined GP33/34-specific response in 100% (12/12) of mice examined between 4.5 and 6 days post-infection. This indicates that although the difference in immunodominance is not large, it is highly consistent. At 8 days post-infection however, the NP396-specific T cell response moved from intermediate dominance (i.e., higher than GP276 and NP205, but lower than GP33/34) to the expected codominant position with the GP33/34-specific response (Murali-Krishna et al., 1998). The combined GP33/34-specific T cell to NP396-specific T cell ratio shifted significantly from 2.0 ± 0.24 at 4.5 days post-infection to 1.2 ± 0.17 (i.e., codominant) at 8 days post-infection ($P = 0.002$). During the memory phase (>60 days post-infection), the NP396-specific T cell response maintained codominance with GP33/34-specific T cell responses or demonstrated a slightly higher frequency than their GP33/34-specific counterpart in 10/14 (71%) of the animals examined.

The shift in immunodominance was even more dramatic between the subdominant T cell populations specific for NP205 and GP276, which switched positions within the hierarchy during the first 8 days after LCMV infection. The NP205-specific response was 3-fold higher than the GP276-specific response at 4.5 and 5 days after infection (Fig. 1) but lost its dominant position over GP276 by 8 days post-infection. Accordingly, the GP276-specific T cell to NP205-specific T cell frequencies shifted from a ratio of 0.29 ± 0.37 to 1.4 ± 0.55 between day 4.5 and day 8 post-infection, respectively ($P = 0.003$). At later time points during the memory phase, the GP276-specific T cell response became even more dominant, with a frequency that was about 3-fold higher than that observed with NP205-specific T cell populations. LCMV-specific CD8$^+$ T cell responses declined between 8 and 15 days post-infection and then remained stable for at least 315 days after infection (Homann et al., 2001; Murali-Krishna et al., 1998). The rapid evolution of the immunodominance hierarchy observed in the spleen during acute LCMV infection indicates that the size of the peptide-specific CD8$^+$ T cell populations identified

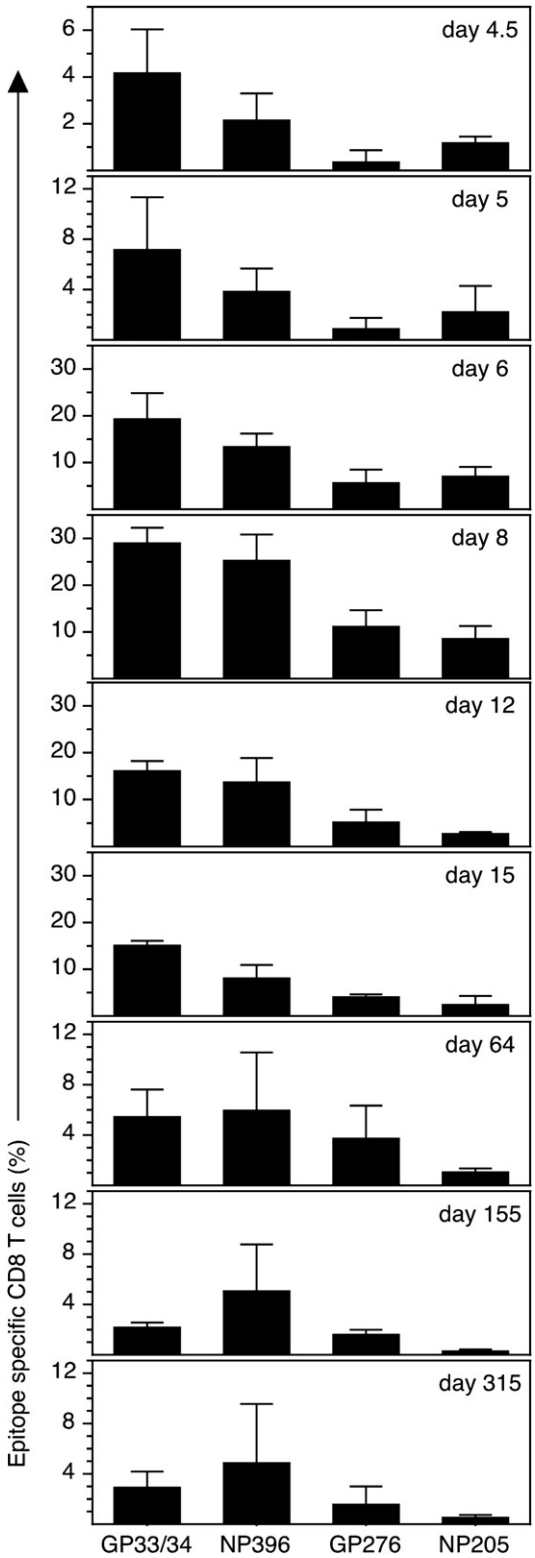

Fig. 1. Evolution of splenic CD8$^+$ T cell immunodominance during acute LCMV infection. The immunodominance hierarchy of representative virus-specific CD8$^+$ T cell responses were examined in the spleen at time points ranging from 4.5 days to 315 days after LCMV infection. CD8$^+$ T cells were stimulated with 1×10^{-4} M peptide (GP33/34, NP396, GP276, or NP205) in the presence of brefeldin A for 6 h directly *ex vivo* and peptide-specific responses were measured by intracellular staining for IFNγ. Similar results were observed when 1×10^{-5} M peptide was used for stimulation (data not shown). The graphs show the average ± standard deviation for each peptide-specific response at the time points indicated. Data represent 3–6 mice per group from 3 to 5 independent experiments.

early after infection (i.e., day 4.5 and day 5) did not necessarily determine the eventual immunodominance hierarchy observed during the peak or memory phase of the virus-specific T cell response.

Functional avidity after infection with LCMV

To assess changes in the functional avidity of the peptide-specific T cell responses, $CD8^+$ T cells obtained at the indicated time points after LCMV infection were stimulated with graded doses of peptide antigen. After stimulation, the magnitude of the $CD8^+$ T cell response at each concentration of peptide was measured using intracellular staining for IFNγ and is expressed as a percentage of the maximum response observed after stimulation with 1×10^{-4} M peptide (Fig. 2a). To determine the magnitude of functional avidity maturation, the concentration of peptide at which 50% of the maximal response was reached was calculated by linear interpolation and normalized to the fold increase over the average functional avidity observed at 4.5 days after infection (Fig. 2b). Between 4.5 and 6 days after infection, the functional avidity of the combined GP33/34-specific response increased by about 4-fold whereas the GP276-specific response showed no observable improvement in peptide sensitivity. In sharp contrast, the NP-specific T cell responses were much more dynamic. NP396-specific T cells demonstrated a 10- to 11-fold increase in functional avidity between 4.5 and 8 days post-infection and peptide sensitivity improved further, with an 80- to 130-fold improvement in functional avidity noted during the memory phase of the response. The functional avidity of NP205-specific $CD8^+$ T cells increased by approximately 30-fold during the acute phase of infection and remained stable for >300 days after infection. Despite their rapid and substantial increase in functional avidity, NP205-specific T cells remained a subdominant T cell population. In contrast, despite only minor changes in functional avidity, GP33/34-specific T cells maintained their immunodominant status. Comparison of immunodominance profiles (Fig. 1) and changes in functional avidity (Fig. 2) indicated that the immunodominance hierarchy of virus-specific $CD8^+$ T cells is not necessarily determined by changes in peptide sensitivity during the course of acute LCMV infection.

Role of CD8 engagement in T cell activation

Engagement of the CD8 coreceptor by MHC Class I is important for $CD8^+$ T cell activation (Bachmann et al., 1999; Purbhoo et al., 2001; Slifka and Whitton, 2001; Viola et al., 1997) and we have previously shown that high functional avidity correlates with resistance to blockade of CD8/MHC Class I interactions by NP118-specific T cells from LCMV-infected BALB/c mice (Slifka and Whitton, 2001). To determine if similar results would be found in LCMV-infected C57BL/6 mice, $CD8^+$ T cells obtained at 5 or 8 days post-LCMV infection (representing low functional avidity or high functional avidity T cell populations, respectively) were stimulated with 1×10^{-5} M GP33/34 or NP396 peptide in the presence or absence of graded doses of anti-CD8 blocking antibody. The magnitude of the response at each concentration of anti-CD8 was determined by intracellular staining for IFNγ and expressed as a percentage of the response observed in the absence of anti-CD8 blockade (Fig. 3a). Low concentrations (e.g., 0.1 μg/ml) of anti-CD8 antibody had little or no effect on GP33/34-specific (Fig. 3b) or NP396-specific (Fig. 3c) $CD8^+$ T cells. In contrast, higher concentrations of blocking antibody significantly inhibited T cells with a low relative functional avidity phenotype (day 5) compared with T cells of the same peptide specificity that had a higher functional avidity phenotype (day 8). The magnitude of the differences in resistance to anti-CD8 blocking antibody reflected the fold-differences in functional avidity; GP33/34-specific responses underwent less avidity maturation between day 5 and day 8 post-infection (Fig. 2) and demonstrated comparatively smaller (but still statistically significant) differences in resistance to anti-CD8 blocking antibody. In contrast, NP396-specific T cells underwent a larger change in functional avidity maturation between 5 and 8 days post-infection and likewise showed a larger difference in resistance to anti-CD8 blockade. The ratio of CD8α/CD8β expression may also be associated with changes in functional avidity (Kroger and Alexander-Miller, 2007b) but we did not find consistent differences in CD8α/CD8β expression that were predictive of higher functional avidity, at

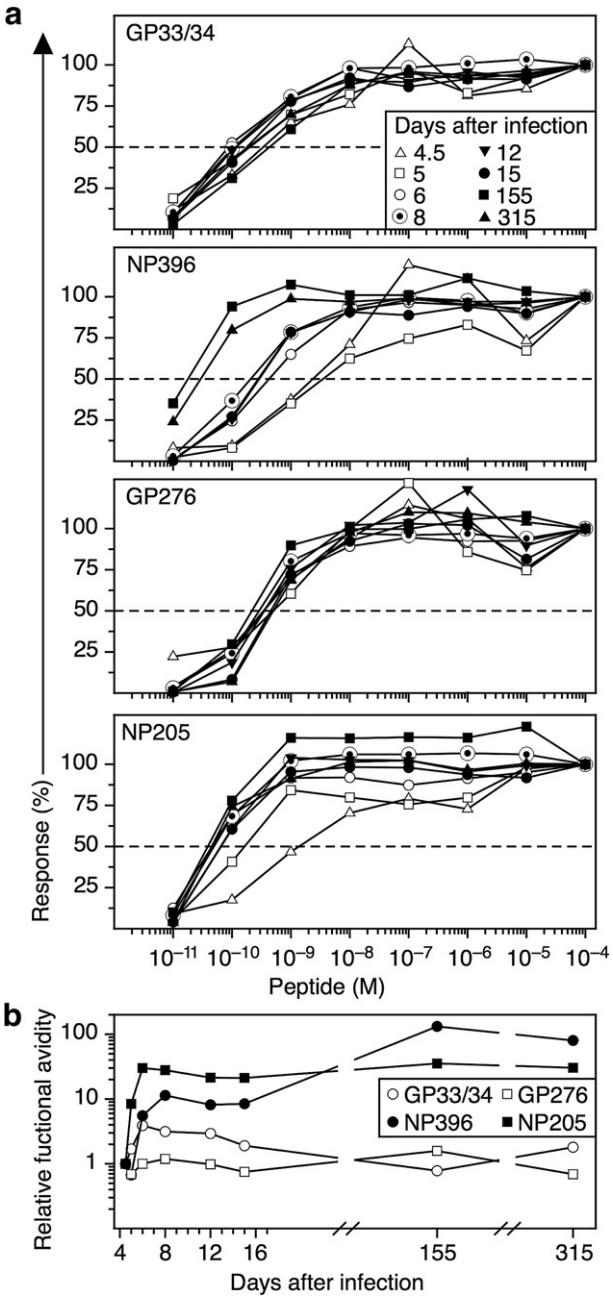

Fig. 2. Functional avidity maturation of splenic $CD8^+$ T cells after LCMV infection. (a) Analysis of functional avidity was performed at the indicated time points after LCMV infection by stimulating T cells with graded doses of GP33/34, NP396, GP276, or NP205 peptide. Peptide-specific $CD8^+$ T cell responses were measured using intracellular staining for IFNγ and expressed as a percentage of the maximum response attained with saturating peptide concentrations (1×10^{-4} M). (b) To normalize the rates of functional avidity maturation among different peptide-specific T cell populations, the peptide concentration at which 50% maximum response was reached at each time point was calculated by linear interpolation and expressed relative to the peptide concentration at which 50% maximum response was reached by the earliest T cells measured at 4.5 days after LCMV infection. The data show the average of 3–6 mice per group from 3 to 5 independent experiments.

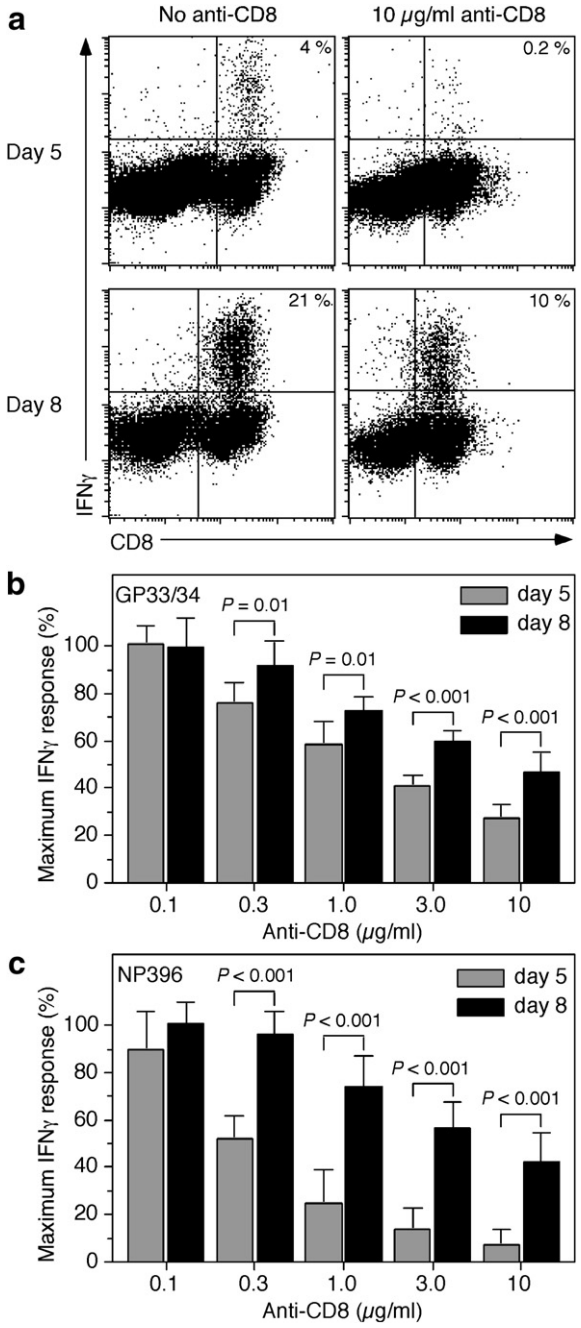

Fig. 3. High functional avidity correlates with resistance to anti-CD8 blockade. To determine the requirement for CD8 coreceptor binding during peptide recognition and T cell activation, $CD8^+$ T cells obtained at 5 or 8 days post-LCMV infection (representing low and high functional avidity populations, respectively) were stimulated with 1×10^{-5} M NP396 or GP33/34 in the presence of graded doses (10–0.1 μg/ml) of anti-CD8 blocking antibodies. Antigen-specific T cell responses were quantified by intracellular staining for IFNγ. (a) Representative dot plots show $CD8^+$ T cell responses following stimulation with NP396 after incubation in the presence or absence of 10 μg/ml anti-CD8 blocking antibodies, numbers in the upper right corners indicate the percentage IFNγ producing cells in the $CD8^+$ T cell population after subtraction of background IFNγ production (<1%). The effect of graded doses of anti-CD8 on low and high functional avidity GP33/34-specific T cells (b) or NP396-specific T cells (c). Data are expressed as a percentage of the maximum response observed in the absence of anti-CD8. *P* values were derived using the two-tailed unequal-variance Student *t*-test. The data show the average ± standard deviation for 7 mice per group from 3 independent experiments.

least within the context of these studies (data not shown). The results in Fig. 3 show that $CD8^+$ T cells with high functional avidity are less dependent on CD8/MHC Class I interactions than low functional avidity T cells. Moreover, statistically significant differences in resistance to anti-CD8 blockade were observed even under circumstances in which the differences in peptide sensitivity were as small as 3- to 5-fold between one T cell population and the other (e.g., GP33/34). This indicates that anti-CD8 blockade is a useful method for determining the functional attributes of T cell populations of the same peptide specificity but which differ in terms of their overall sensitivity to peptide stimulation.

Pharmacological inhibition of Src kinase activity

The anti-CD8 blocking antibody experiments provided evidence supporting the theory that improvements in signal transduction through the TcR may contribute to increased functional responsiveness in high avidity T cells. However, these studies fall short on providing a molecular mechanism for the increase in TcR signaling efficiency in highly responsive T cell populations. The cytoplasmic tails of CD4 and CD8 coreceptors associate with Src kinases such as Lck and Fyn (Barber et al., 1989; Rudd et al., 1991, 1988; Veillette et al., 1988), important signal transduction proteins involved in the initiation and regulation of T cell activation (Bachmann et al., 1999; Latour and Veillette, 2001; Palacios and Weiss, 2004; Salmond et al., 2009; Weiss, 1993; Zamoyska et al., 1989). To determine if high functional avidity was associated with enhanced activity of Src kinase family members, $CD8^+$ T cells with a low or high functional avidity phenotype (obtained at 5 or 8 days post-infection, respectively) were stimulated with 1×10^{-5} M of the indicated peptide in the presence or absence of graded doses of the Src kinase inhibitor, PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) (Hanke et al., 1996; Karni et al., 2003). To confirm the results obtained with PP2, we also tested a second Src kinase inhibitor, 7C-PP (Cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d]pryrimidin-4-ylamine) (Arnold et al., 2000; Burchat et al., 2000; Calderwood et al., 2002). Peptide-specific $CD8^+$ T cells of low functional avidity were significantly more susceptible to inhibition by PP2 or 7C-PP than T cells of high functional responsiveness (Fig. 4). In contrast, there was virtually no effect on antigen-specific IFNγ production following incubation with 10 μM PP3 (4-amino-7-phenylpyrazol[3,4-d]pyrmidine), a compound similar to PP2 but without Src kinase-specific inhibitory properties (data not shown). Similar to our anti-CD8 blockade experiments (Fig. 3), we found significant correlations between increased functional avidity and increased resistance to pharmacological inhibition of T cell activation (Fig. 4). In terms of peptide-specific T cell activation in the presence of 1 μM PP2 or 2 μM PP2 (or 0.25 μM to 0.5 μM 7C-PP), low avidity T cells obtained at 5 days post-LCMV infection were consistently more susceptible to inhibition than high avidity T cells of the same peptide specificity obtained at 8 days post-infection. This result corresponds well with their peptide-specific thresholds of activation (Fig. 2). PP2 is a potent and selective inhibitor of Src kinases and is typically used at a concentration of 10 μM to completely abrogate Src kinase activity. Not surprisingly, we found that T cell activation was completely blocked at this dose, indicating that T cells of high functional avidity were similar to T cells of low functional avidity in that they rely heavily on Src kinase activity for effective signal transduction and were unable to compensate for the loss of Src kinase activity by utilizing other families of kinases. Similar to anti-CD8 blockade, these pharmaceutical inhibitors of kinase activity provide a useful tool for determining the quality/functional capacity of T cell populations assessed at different stages of maturity.

Discussion

In these studies, we measured the frequency and functional responsiveness of representative $CD8^+$ T cell populations specific for LCMV peptides: NP205, NP396, GP276 and the average response to GP33/34 (an H-$2K^b$ and H-$2D^b$-restricted peptide) in C57BL/6 mice during the course of acute LCMV infection. We found that

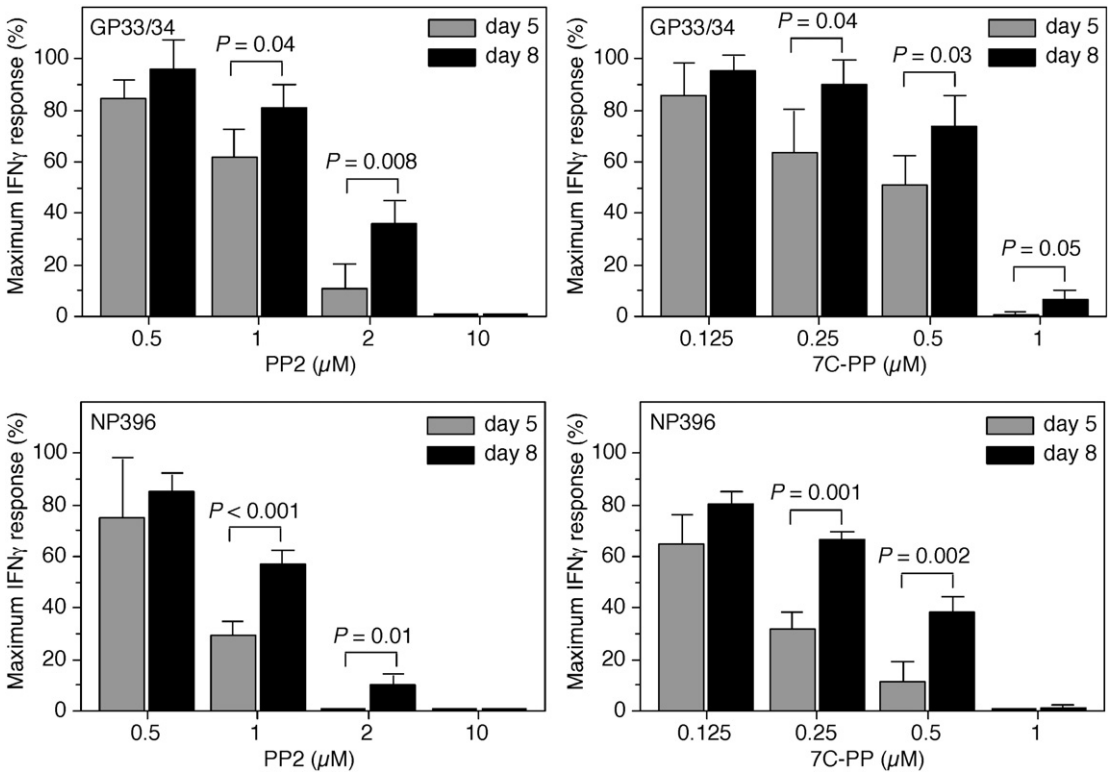

Fig. 4. Inhibition of Src kinase activity in T cells with a high or low functional avidity phenotype. The relationship between Src kinase activity and T cell responses to peptide stimulation was examined by measuring IFNγ production by $CD8^+$ T cells obtained at 5 or 8 days post-LCMV infection after stimulation with 1×10^{-5} M GP33/34 or NP396 peptide in the presence or absence of the indicated concentrations of Src kinase inhibitor. Data are expressed as the percentage of the maximum response observed in the absence of Src kinase inhibitors. *P* values were derived using the two-tailed unequal-variance Student *t*-test. The data show the average ± standard deviation for 4 mice per group from 2 independent experiments and are representative of 5 independent experiments.

immunodominance profiles were not static, but instead underwent a previously unrecognized shift in the dominance hierarchy during the first 8 days after acute viral infection. This is important because it indicates that although naïve peptide-specific T cell precursor frequencies can greatly influence the initial immunodominance pattern, other factors must also be involved with determining the final dominance hierarchy that develops during acute LCMV infection. Further analysis revealed that modifications in immunodominance occurred independently from changes in functional avidity maturation as measured by peptide dose–response curves and resistance to inhibition with anti-CD8 blocking antibody or the pharmacological Src kinase inhibitors, PP2 and 7C-PP. Together, these results indicate that immunodominance is complex and although the naïve T cell repertoire can influence initial immunodominance profiles, there is much yet to be learned about the rules that govern the final immunodominance hierarchy elicited following acute viral infection.

Shifting patterns of T cell immunodominance have been observed in several models of chronic viral infection including EBV (Woodberry et al., 2005), SIV (Edwards et al., 2002), murine CMV (Munks et al., 2006), MHV (Bergmann et al., 1999), and chronic LCMV infection (Fuller and Zajac, 2003; Ou et al., 2001; van der Most et al., 2003, 1996; Wherry et al., 2003; Zajac et al., 1998). Following chronic LCMV infection, it is striking that T cell responses directed against GP epitopes are typically maintained whereas the dominant NP396-specific T cell response is preferentially lost. This may be due to differences in the kinetics (Probst et al., 2003) or quantity/duration (Buchmeier and Zajac, 1999; Wherry et al., 2003) of NP versus GP expression *in vivo*. For instance, LCMV NP mRNA is transcribed directly from genomic RNA whereas GP mRNA synthesis occurs only after genomic RNA is replicated. This results in more rapid expression of NP mRNA compared to GP mRNA (Fuller-Pace and Southern, 1988) and in turn, results in more rapid expression of viral peptides on the surface of infected cells (Probst et al., 2003). If the NP-specific T cells are triggered more rapidly than GP-specific T cells, then it is possible that cytokines [e.g., IFNγ (Rodriguez et al., 2002)] produced during the NP-specific response may alter subsequent T cell responses to GP-specific epitopes. It is unclear if this might account for the differences in functional avidity maturation, but it is interesting that $CD8^+$ T cell responses against NP118, NP396, and NP205 undergo substantial improvements in functional avidity during the early course of acute LCMV infection whereas responses against GP33/34 and GP276 undergo much smaller changes in peptide sensitivity. In terms of immunodominance, NP396-specific T cell responses outcompete the combined GP33/34 response, but GP276 quickly outcompetes the NP205-specific T cell response, indicating that simple differential expression of GP and NP protein cannot be solely responsible for determining immunodominance profiles.

Multiple factors are involved with the evolution of an antigen-specific T cell response including early selection of high affinity T cell populations (Busch and Pamer, 1999; McHeyzer-Williams et al., 1999; Savage et al., 1999; Zehn et al., 2009) followed by further changes in functional responsiveness to peptide stimulation (Kroger and Alexander-Miller, 2007a; Slifka and Whitton, 2001) that appear to involve improvements in Src kinase activity (Fig. 4). There are several ways to measure and compare the quality and functional attributes of antigen-specific T cell populations. Peptide/MHC tetramers can be used to measure structural avidity (i.e., the binding of the TcR to peptide/MHC molecules) but this does not always predict functional attributes since chronically infected mice can have LCMV-specific T cells that clearly bind peptide/MHC but remain unresponsive to peptide stimulation (Wherry et al., 2003; Zajac et al., 1998). Another approach is to measure peptide sensitivity or "functional avidity" by stimulating T cells with graded doses of peptide antigen and determining their ability to respond to low levels of antigenic stimulation. Functional

avidity measurements take into account the combined effects of structural avidity and functional responsiveness. In our studies, we used peptide-induced IFNγ production to measure functional avidity but measurements of other cytokines (e.g., TNFα, IL-2, etc.) or cytolytic activity against peptide-coated targets may also be employed. In a previous work, we have shown that functional avidity based on IFNγ production is similar to that observed for TNFα or CTL activity following acute LCMV infection (Slifka and Whitton, 2001). T cells with a high functional avidity phenotype have been shown to be more protective against viral infection than T cells of a low functional avidity phenotype (Alexander-Miller, 2005; Alexander-Miller et al., 1996). Improvements in functional avidity can also occur in TcR transgenic T cells (Kroger and Alexander-Miller, 2007a; Slifka and Whitton, 2001). We found up to a 10-fold increase in functional avidity in GP33-specific T cells from LCMV-infected P14 mice at 8 days post-infection (Slifka and Whitton, 2001) and in subsequent studies with P14 mice, P14 mice crossed with $Rag^{-/-}$ mice, or in adoptive transfer experiments we found a more modest 3- to 4-fold improvement in functional avidity by 8 days post-infection (data not shown) and this is similar to the GP33/34-specific T cell responses observed in wild type C57BL/6 mice (Fig. 2b). This indicates that although the studies described in this report represent the combined average of the GP33 (H-$2D^b$)-specific and GP34 (H-$2K^b$)-specific $CD8^+$ T cell responses, the results appear to be similar to that observed when only GP33 (H-$2D^b$)-specific T cells are examined. An elegant study using mice with Lck expressed under an inducible promoter has clearly shown that Lck is not required for GP33-specific memory T cell responsiveness to peptide antigen following acute LCMV infection (Tewari et al., 2006). One explanation for these findings is that it is possible that Lck may not be involved with functional responsiveness in T cell populations that do not undergo a large degree of functional avidity maturation. It will be important to learn whether Lck is also not required for NP396-specific T cell responsiveness to peptide antigen (i.e., T cells that undergo large changes in functional avidity) or if these T cell populations differ with respect to their use of Lck or perhaps other Src kinases. Interestingly, NP396-specific T cell responses in C57BL/6 mice underwent the largest degree of functional avidity maturation during primary infection (Fig. 2b) and become the most dominant virus-specific T cell population observed following re-infection (Murali-Krishna et al., 1998; Tebo et al., 2005). Similar shifts in immunodominance are observed following acute infection with influenza virus; during the primary response, the PA224-specific and NP366-specific T cell responses are codominant whereas following secondary infection, the T cell response is sharply dominated by NP366-specific $CD8^+$ T cells (Belz et al., 2001, 2000). It is yet unclear if this shift in immunodominance during secondary infection is due, at least in part, to improved functional avidity within a peptide-specific T cell population that develops during the primary immune response or if it may be due to a variety of other factors such as T cell precursor frequency and competition during re-infection (La Gruta et al., 2006a,b).

We have previously demonstrated that resistance to anti-CD8 blocking antibodies is a simple and effective approach for distinguishing between peptide-specific T cell populations with low or high avidity (Slifka and Whitton, 2001) (Fig. 3). In these current studies, we have broadened this type of functional analysis to include peptide stimulation in the presence or absence of Src kinase inhibitors such as PP2 and 7C-PP (Fig. 4). These pharmacological inhibitors of signal transduction provide a mechanistic approach to determining the functional capacity of virus-specific T cells. In this regard, we found that T cells obtained at 8 days post-LCMV infection (representing a high functional avidity population) were significantly more resistant to PP2 and 7C-PP inhibition than T cells examined at 5 days post-LCMV infection (representing a low functional avidity population). By using two distinct pharmacological inhibitors of Src kinase activity, PP2 and 7C-PP, we are able to measure T cell function by two independent biochemical approaches. In previous studies, we found changes in Lck expression that were linked to increased T cell responsiveness based on intracellular staining with polyclonal anti-Lck antibodies (Slifka and Whitton, 2001). However, similar to another study (La Gruta et al., 2006a,b), we did not find substantial differences in the total cellular expression of T cell-associated Src kinases, Lck or Fyn by flow cytometry using monoclonal antibodies specific to these antigens (data not shown). These results do not necessarily rule out a role for improved signal transduction in the development of improved functional avidity as it is possible that changes in subcellular localization and/or the activation state of these kinases, rather than total cellular levels, are more important for improved T cell signaling through the TcR. Previous studies have indicated a higher association of Lck with the cytoplasmic tail of CD8 molecules following LCMV infection (Bachmann et al., 1999) and this may represent at least one mechanism responsible for improved T cell responses and functional avidity maturation observed during the early stages of acute viral infection. In addition to standard peptide dose–response curves and anti-CD8 antibody blockade, we show here that exposure to pharmacological Src kinase inhibitors such as PP2 and 7C-PP can be used to characterize T cell function and distinguish between high and low avidity T cell populations of the same antigen specificity. This may be a useful approach for assessing the quality of vaccine-induced $CD8^+$ T cell populations.

Conclusions

The results of this study indicate that shifting immunodominance profiles are not limited to chronic LCMV infection and instead can be observed during the very early stages of acute LCMV infection as well. Our studies focused on T cell immunodominance in the spleen (a major site of LCMV infection) and it is possible that the rules for immunodominance differ in other anatomical sites. These early changes in $CD8^+$ T cell immunodominance observed in the spleen suggest that the naïve T cell precursor frequency is not the sole factor involved with determining the immunodominance hierarchy since the relative magnitude of different peptide-specific T cell populations continue to evolve during the first 4–8 days after infection. Analysis of functional avidity maturation of four representative peptide-specific T cell populations indicated that immunodominance profiles evolved independently of changes in peptide sensitivity. Interestingly, not all T cell populations undergo measurable functional avidity maturation and T cells specific for the LCMV glycoprotein (GP) undergo much less avidity maturation than T cells specific for the LCMV nucleoprotein (NP). This may have further implications on T cell immunobiology since chronic LCMV infection is known to delete or functionally "exhaust" NP-specific T cells over GP-specific T cell populations. Together, our results demonstrate that immunodominance is not static during acute LCMV infection and instead evolves quickly during the early stages of infection before reaching a stable pattern of immunodominance that is maintained during the memory phase of the antiviral immune response.

Methods

Mice and virus

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine), C57BL/6.Thy1^{a}IgH^{a}Gpi^a mice were bred at OHSU and used interchangeably with C57BL/6 mice. Animals were infected intraperitoneally with 2×10^5 PFU LCMV-Armstrong (Arm-53b) at 5–12 weeks of age. The Oregon Health & Science University IACUC committee approved all animal protocols.

Reagents

HPLC-purified (>95% pure) GP33/34 (KAVYNFATM), NP396 (FQPQNGQFI), GP276 (SGVENPGGYCL), and NP205 (YTVKYPNL) peptides were purchased from Alpha Diagnostic International (San Antonio, TX), Aves Labs (Tigard, OR) and Sigma Genosys (St Lois, MO). Anti-CD8 blocking antibody (clone 53-6.7) was purchased from PharMingen (San Diego, CA). Src kinase inhibitors, PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) (Hanke et al., 1996; Karni et al., 2003) and 7C-PP (Cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d]pryrimidin-4-ylamine) (Arnold et al., 2000; Burchat et al., 2000; Calderwood et al., 2002), were purchased from EMD biosciences (Calbiochem, San Diego, CA) and Sigma-Aldrich (St Lois, MO) respectively, and dissolved at 5 mM in DMSO.

Direct ex vivo stimulation and intracellular staining

Peptide stimulation and intracellular cytokine staining was performed as described previously (Raue and Slifka, 2007). In summary, single cell splenocyte suspensions were stimulated with the indicated concentrations of peptide in RPMI supplemented with 5% FBS (Hyclone, Logan, UT), 20 mM HEPES, L-glutamine, antibiotics and 2 μg/ml Brefeldin A (Sigma) at 37 °C, 6% CO_2 for 6 h. After stimulation, cells were stained overnight with anti-CD8α (clone 5H10, Caltag, Burlingame, CA) and fixed in PBS + 2% formaldehyde the next morning. After staining with anti-IFNγ (Caltag) in Permwash (0.1% saponin (Sigma), 0.1% NaN_3 (Sigma), 2% FBS in PBS) at 4 °C cells were washed with Permwash and PBS + 1% FCS and resuspended in PBS + 2% formaldehyde for analysis. Data was acquired on a FACSCalibur or LSR-2 and analyzed using CellQuest (Becton Dickinson, San Jose, CA) or FlowJo software (Treestar, Ashland, OR). Non-specific IFNγ production after incubation with medium alone was subtracted to yield the frequency of virus-specific $IFN\gamma^+$ $CD8^+$ T cells. For inhibition experiments, cells were pre-incubated with anti-CD8 blocking antibody (clone 53-6.7 used at 4 °C) or the Src kinase inhibitors, PP2 or 7C-PP (at 37 °C) for 20 min prior to addition of peptide plus Brefeldin A and incubated at 37 °C for 6 h.

Statistics

Statistical significance was determined using a two-tailed Student's *t*-test with unequal variance (Excel, Microsoft, WA). *P* values of ≤ 0.05 were considered statistically significant.

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